Vol. 134, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1986 Pages 240-247

NONMUSCLE MYOSIN PHOSPHORYLATION SITES FOR CALCIUM-DEPENDENT AND CALCIUM-INDEPENDENT PROTEIN KINASES

TOMMY C. HASSELL, BRUCE E. KEMP* AND RUTHANN A. MASARACCHIA

DEPARTMENT OF BIOCHEMISTRY
NORTH TEXAS STATE UNIVERSITY
DENTON, TX 76203

*DEPARTMENT OF MEDICINE
UNIVERSITY OF MELBOURNE
REPATRIATION GENERAL HOSPITAL
HEIDELBERG, VICTORIA 3081, AUSTRALIA

Received November 11, 1985

Thymus myosin, light chains and a synthetic peptide (S-S-K-R-A-K-A-K-T-T-K-K-R-P-Q-R-A-T-S-N-V-F-S) corresponding to the N-terminal sequence of smooth muscle myosin light chains were compared as substrates for calcium/calmodulin-dependent protein kinase (MLCK), calcium/phospholipid-dependent protein kinase (PKC), and a MgATP-activated protein kinase (H4PK) from lymphoid cells. All protein kinases catalyzed phosphorylation of the substrates although H4PK showed higher affinity for isolated light chains and the peptide. Phosphoamino acid analysis and analysis of thermolysin peptides established that PKC catalyzed phosphorylation of threonine-9 or 10. In addition, PKC and H4PK catalyzed phosphorylaton at serine-19, the MLCK site. Collectively the data support the hypothesis that myosin filament assembly in nonmuscle cells may be regulated by a variety of calcium-dependent and calcium-independent protein kinases.

Phosphorylation of myosin light chains in smooth muscle and nonmuscle cells is catalyzed by the calcium/calmodulin-dependent myosin light chain kinase (1), the calcium/phospholipid-dependent protein kinase (2,3) and a calcium-independent, protease-activated protein kinase (4-6). The MLCK-catalyzed phosphorylation promotes activation of actin-activated MgATPase activity (1,7) and myosin filament formation (8) in smooth muscle and nonmuscle cells. This enzyme exhibits a markedly restricted substrate

Abbreviations: MLCK, calcium/calmodulin-dependent protein kinase; PKC, calcium/phospholipid dependent protein kinase; H4PK, calcium-independent, MgATP-activated protein kinase; MLC(1-23), S-S-K-R-A-K-A-K-T-T-K-K-R-P-Q-R-A-T-S-N-V-F-S; H4(Ser 47), V-K-R-I-S-G-L; SDS PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate; TLE, thin layer electrophoresis; PS, phosphatidylserine.

0006-291X/86 $1.50

specificity and requires calcium-calmodulin for activity (1,2). In contrast, PKC and H4PK have been shown to catalyze the phosphorylation of multiple substrates, including histones H1 and H4 and the ribosomal protein S6 (9-12).

The substrate specificity determinants for each of these phosphotransferases has been investigated (13-17). Kemp and coworkers (13,14) have demonstrated that lysyl and arginyl residues located in the myosin light chain sequence K-K-R-P-Q-R-A-T-S-N-V-F were the principal determinants for MLCK reactivity. Masaracchia and coworkers (15) systematically investigated the reactivity of H4PK with synthetic peptides derived from the sequence V-K-R-I-S-G-L. A strict requirement for the dibasic -K-R- sequence was demonstrated. O'Brian et al (16) reported that the synthetic sequence R-R-K-A-S-G-P-P-V was phosphorylated with a K_{mapp} = 130 μM by PKC although other data have suggested threonyl residues may be preferred modification sites for PKC (3). Ferrari et al (17) reported phosphorylation of several basic peptides by PKC although no discrete specificity determinants for this enzyme have been established.

In this report a synthetic peptide containing the seryl residue modified by MLCK in the gizzard myosin light chain sequence has been used to investigate the PKC and H4PK reactivity with myosin light chains.

MATERIALS AND METHODS

MLCK, PKC and H4PK were prepared from murine lymphosarcoma P1798 as described previously (4,11,18,19). Bovine trachea MLCK was a generous gift from Dr. James T. Stull (University of Texas Health Science Center, Dallas, TX). Protein kinase assays were conducted for 10 min at 30° unless otherwise noted. The assay was conducted in the presence of 20 mM MES, pH 6.8, 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (50-200 dpm/pmol) and substrates and effectors as noted in a total volume of 50 μl. When present, the concentrations of effectors were as follows: $CaCl_2$, 1 mM; calmodulin, 30 μg/ml; PS, 90 μg/ml. Phosphorylated peptide and proteins were quantitated by the method of Glass et al. (20). Histones and [γ-^{32}P]ATP were prepared as described previously (4); calmodulin was purified from bovine testes (21) and PS was obtained from Supelco. In some experiments myosin light chain phosphorylation was analyzed by SDS PAGE (15% gel) and autoradiography as previously described (12).

Thymus myosin was prepared according to the KI procedure of Scholey et al. (22), and thymus myosin light chains were extracted from the myosin (23). Actin-activated myosin ATPase was assayed according to Trotter and Adelstein (24). The synthetic peptide corresponding to the N-terminal 23 residues of chicken gizzard regulatory myosin light chain was synthesized and purified as described previously (13, 14). Phosphamino acids and peptides were separated by TLE (15).

RESULTS

Purified thymus myosin and thymus myosin light chains were phosphorylated by MLCK, PKC and H4PK (Figure 1). With both substrates, SDS-PAGE demonstrated that the principal phosphorylated protein was the Mr 23,000 light chain (LC_R). MLCK and H4PK phosphorylated a second protein (Mr = 20,000) in some preparations. Since the occurrence of this protein is variable, it is likely that it represents a proteolytic product of LC_R. No endogenous kinase activity was detected with either myosin or light chains incubated with MgATP in the absence of exogenous protein kinase.

Previous data have established the effects of MLCK and PKC-catalyzed phosphorylation on myosin ATPase activity (1-3). MLCK catalyzes an activation of the myosin MgATPase, but prior PKC treatment diminishes the MLCK effect (3). H4PK-catalyzed phosphorylation of thymus myosin resulted in a 10-fold increased MgATPase activity to a value of 630 nmol P_i hydrolyzed/ 10 min-mg myosin. Comparable MgATPase activation was observed with actomyosin. In both cases the increased activity was not inhibited by 1 mM EGTA.

When MLCK, PKC and H4PK concentrations were adjusted to give comparable activity towards myosin in all assays, the increase in catalytic rate observed

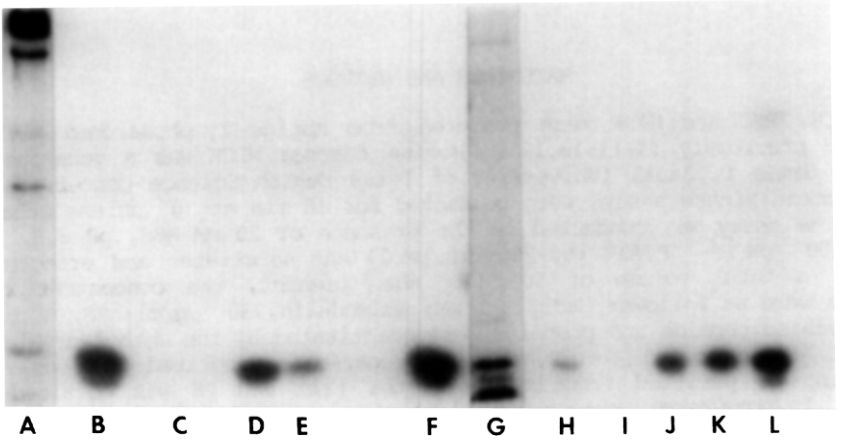

Figure 1. Phosphorylation of thymus myosin and isolated light chains by calcium-dependent and calcium-independent protein kinases. Purified thymus myosin (A-F; 70 µg) or myosin light chains (G-K; 20 µg) were incubated for 10 min with Mg [γ-^{32}P]ATP and bovine trachea MLCK in the presence (B,H) or absence (C,I) of calcium/calmodulin, PKC in the presence (D,J) or absence (E,K) of calcium PS, or H4PK (F,L) as described in Methods. Stained samples (A,G) and autoradiographs (B-F;H-L) of the SDS-PAGE analysis are shown.

Table 1. Comparative rates of myosin, light chain and peptide phosphorylation by MLCK, PKC and H4PK

Enzymes	Protein Kinase Activity (pmol/10 min)		
	Myosin	Light Chain	MLC(1-23)
MLCK	1.3	<1.0	<1.0
+CaCalmodulin	5.8	3.0	11.3
PKC	3.5	6.0	<1.0
$+Ca^{+2}$,PS	7.0	5.5	597
H4PK	6.4	49	2900

Enzymes (MLCK 0.9 μg; PKC 0.4 μg; H4PK 10 μg) were incubated with myosin (100 μg) light chains (23 μg) or MLC(1-23) (435 μM) as described in Figure 1. Protein phosphorylation was quantitated according to Glass et al. (19). All enzymes were purified from lymphosarcoma.

with light chains or the peptide MLC(1-23) substrates was not comparable for the three enzymes (Table 1). With MLCK, a two-fold increase over the myosin rate was observed when the peptide was used as substrate. With all three substrates the activity was dependent on added calcium and calmodulin. The MLC(1-23) was phosphorylated 85-fold faster than myosin by PKC. Furthermore, the peptide phosphorylation was totally dependent upon added calcium and phosphatidylserine whereas the reactivity with myosin and light chains demonstrated little effector dependency. With both MLCK and PKC, the activity towards intact myosin and isolated light chains was comparable. H4PK catalyzed light chain and MLC(1-23) phosphorylation at rates 8- and 480-fold, respectively, greater than myosin.

Since the previous data indicated that PKC (3) and a protease-activated kinase, which is highly similar to H4PK (4,5), catalyzed phosphorylation of myosin light chain sites different from those modified by MLCK, further analyses of the phosphorylated residues in MLC(1-23) were carried out. TLE analysis of products obtained by partial acid hydrolysis of MLC(1-23) which was (^{32}P)-labelled with Mg [γ-^{32}P]ATP and H4PK or PKC indicated that H4PK catalyzed phosphorylation of seryl residues, but PKC catalyzed phosphorylation of both seryl and threonyl residues (Figure 2). MLCK catalyzed only serine phosphorylation (data not shown). Since multiple seryl and threonyl residues are present in the MLC(1-23) sequence, the phosphorylated peptide was further analyzed by thermolysin digestion and TLE analysis of (^{32}P)-labelled products (Figure 3).

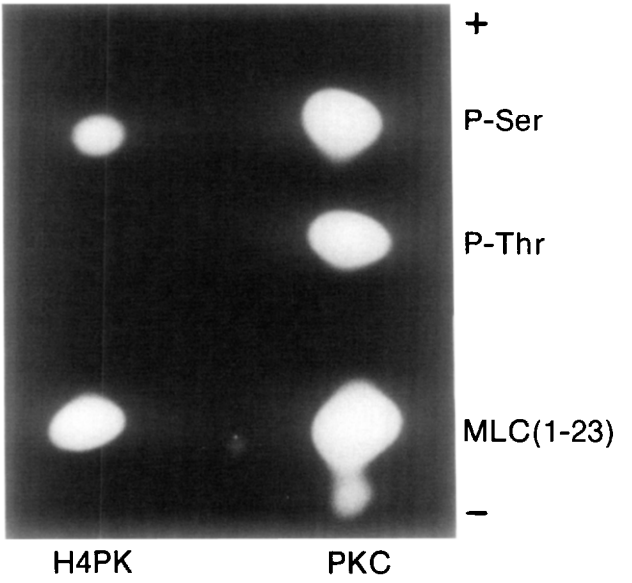

Figure 2. Identification of phosphorylated residues in MLC(1-23) incubated with H4PK and PKC. Following 30 min incubation of MLC(1-23) with H4PK or PKC, the peptide was partially hydrolyzed with 6N HCl for 1 hour at 100°C. Thin layer electrophoresis of the hydrolysate was carried out at 400 volts for 4 hours (pH 1.9) and the phosphorylated products were detected by autoradiography.

The specificity of thermolysin predicts that at pH 6.4 the N-terminal seryl residues (S-1, S-2) will occur in a short (n=5) basic peptide; the T-9 and T-10 residues will occur in a large (n=12) basic peptide; and T-18 and S-19, the MLCK phosphorylation sites (14), will occur in a short (n=4 or 6) neutral peptide. Phosphorylation will increase the acidity of each peptide at pH 6.4. When phosphopeptide obtained from incubation of MLC(1-23) with H4PK or MLCK was digested with thermolysin and analyzed by TLE, the distribution of (^{32}P)-labeled peptides was identical. In both cases, the (^{32}P) phosphate migrated with a slightly acidic peptide, consistent with the assignment of S-19 as the modified residue. In contrast, in the thermolysin digest of the MLC(1-23) incubated with PKC, three major phosphopeptides were detected (Figure 3). Approximately 20% of the radioactivity occurred in a slightly acidic peptide which coelectrophoresed with the H4PK phosphopeptide. Two major basic phosphopeptides which migrated at rates of 3.6 and 8.2 mm/volt/hr were also detected. The specificity of thermolysin predicts that these are peptides 1-17 and 6-17 which contain the T-9 and T-10 residues. The absence of any

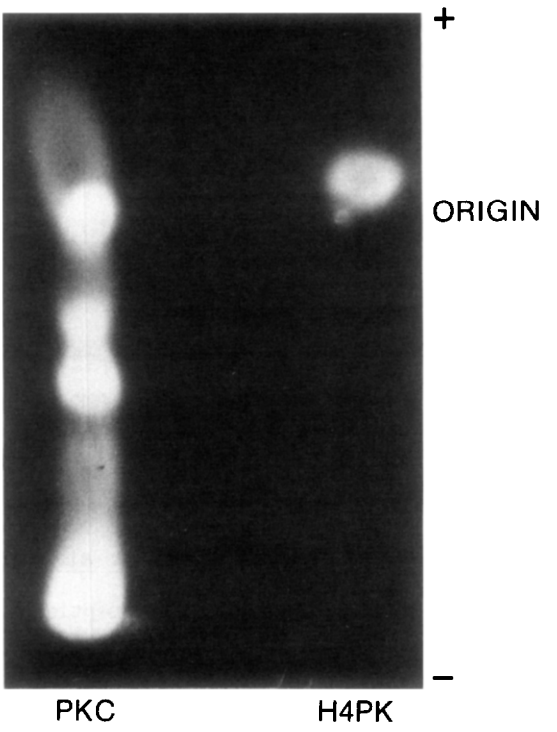

Figure 3. Identification of phosphorylated peptides in MLC(1-23) incubated with H4PK and PKC. Following 30 min incubation of MLC(1-23) with H4PK or PKC, the peptide was digested with 2% thermolysin for 3 hours at 35°. Thin layer electrophoresis of the digested peptide was carried out at 500 volts for 2 hours (pH 6.4). Phosphorylated products were detected by autoradiography.

basic phosphopeptide in the H4PK or MLCK digest indicates that the thermolysin digest was complete and none of the peptides observed in the PKC experiments represent MLC(1-23).

DISCUSSION

Myosin phosphorylation in crude preparations of smooth muscle and nonmuscle cells has been shown to be both calcium-dependent and calcium-independent (25-27). The site modified by MLCK contains multiple basic residues with a specific spatial orientation in the primary structure (13,14). Data presented here establish that PKC and H4PK also catalyze myosin light chain phosphorylation in the N-terminal sequence containing the MLCK specificity determinants. Since serine-19 in the peptide is modified by H4PK and myosin phosphorylation activates the Mg ATPase, the H4PK phosphorylation site appears identical to the MLCK site.

PKC catalyzes phosphorylation of a threonine site which is predicted to be T-8 or T-9 based on the peptide analysis. This is consistent with data obtained with heavy meromyosin (3) which suggest that the PKC and MLCK phosphorylation sites are close enough to permit modulation of the substrate reactivity by multisite phosphorylation, although additional sites may also be modified by PKC.

The specificity determinants for PKC-catalyzed phosphorylation appear to differ substantially from those described for H4PK and MLCK substrates (13-15). For example, threonyl residues are modified at a rate comparable to the seryl residues. Further, basic residues occur both N-terminal and C-terminal to the phosphorylation site. Ferrari et al. (17) also found the most favorable reaction kinetics with synthetic peptides containing basic residues flanking the phosphorylation site on both sides.

Previous data would not predict that the MLC(1-23) sequence Q-R-A-T-S-N-V would serve as a good substrate for phosphorylation by H4PK since no -K-R- sequence is present (15). However, multiple basic residues and a prolyl residue precede this sequence, suggesting that peptide secondary structure may significantly influence the reactivity of this substrate with H4PK. Phosphorylation of this site in myosin is supported by the evidence that actin-activated MgATPase is regulated by the H4PK phosphorylation.

These studies provide further evidence for the phosphorylation of nonmuscle myosin in the N-terminal sequence of the myosin light chain by multiple protein kinases and support the hypothesis that regulation of filament assembly and reactivity in nonmuscle cells may occur in response to multiple physiological signals which selectively activate specific protein kinases.

ACKNOWLEDGMENT

These studies were supported in part by NIH grants GM32350, BRSG S07 RR0 7195 awarded by the Biomedical Research Support Grant Program Division of Research Resources NIH and The Robert A. Welch Foundation B864 to RAM and the National Health and Medical Research Council and Anticancer Council of Victoria to BEK.

REFERENCES

1. Adelstein, R. S. and Eisenberg, E. (1980) Annu. Rev. Biochem. 49, 921-956.
2. Nishikawa, M., Hidaka, H. and Adelstein, R. S. (1983) J. Biol. Chem. 258, 14069-14072.
3. Nishikawa, M., Sellers, J. R., Adelstein, R. S. and Hidaka, H. (1984) J. Biol. Chem. 259, 8808-8814.
4. de la Houssaye, B. A., Eckols, T. K. and Masaracchia, R. A. (1983) J. Biol. Chem. 258, 4272-4278.
5. Masaracchia, R. A. and Michnoff, C. A. (1983) Fed. Proc. 42, 2251.
6. Tuazon, P. T., Stull, J. T. and Traugh, J. A. (1982) Biochem. Biophys. Res. Commun. 108, 910-917.
7. Hartshorne, D. J. and Persechini, A. (1980) Ann. N.Y. Acad. Sci. 356, 130-141.
8. Scholey, J. M., Taylor, K. A. and Kendrick-Jones, J. (1980) Nature (Lond.) 287, 233-235.
9. Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7603-7609.
10. Werth, D. K., Niedel, J. E. and Pastan, J. (1983) J. Biol. Chem. 11423-11426.
11. Magnino, P. E., de la Houssaye, B. A. and Masaracchia, R. A. (1983) Biochem. Biophys. Res. Commun. 116, 675-681.
12. Donahue, M. J. and Masaracchia, R. A. (1984) J. Biol. Chem. 259, 435-440.
13. Kemp, B. E., Pearson, R. B. and House, C. (1983) Proc. Natl. Acad. Sci. USA 80, 7471-7475.
14. Kemp, B. E. and Pearson, R. B. (1985) J. Biol. Chem. 260, 3355-3359.
15. Eckols, T. K., Thompson, R. E> and Masarachia, R. A. (1983) Eur. J. Biochem. 124, 249-254.
16. O'Brian, C. A., Lawrence, D. S., Kaiser, E. T. and Weinstein, I. B. (1984) Biochem. Biophys. Res. Commun. 124, 296-302.
17. Ferrari, S., Marchiori, F., Borin, G., and Pinna, L. A. (1985) FEBS Lett. 184, 72-77.
18. Schatzman, R. C., Raynor, R. L., Fritz, R. B. and Kuo, J. F. (1983) Biochem. J. 209, 435-443.
19. Hathaway, D. R. and Adelstein, R. S. (1979) Proc. Nat. Acad. Sci. USA 76, 1653-1657.
20. Glass, D. B., Masaracchia, R. A., Feramisco, J. and Kemp, B. E. (1978) Anal. Biochem. 87, 566-575.
21. Dedman, J. R., Patten, J. D., Jackson, R. L., Johnson, J. D. and Means, A. R. (1977) J. Biol. Chem. 252, 8415-8422.
22. Scholey, J. M., Smith, R. C., Drenckhahn, D., Groschel-Stewart, U. and Kendrick-Jones, J. (1982) J. Biol. Chem. 257, 7737-7745.
23. Blumenthal, D. K. and Stull, J. T. (1980) Biochemistry 19, 5608-5614.
24. Trotter, J. A. and Adelstein, R. S. (1979) J. Biol. Chem. 252, 878-8785.
25. Scordilis, S. P., Anderson, J. L., Pollack, R., and Adelstein, R. S. (1977) J. Cell Biol. 74, 940-948.
26. Matsumura, S., Murakami, N., Yasuda, S., and Kumon, A. (1982) Biochem. Biophys. Res. Commun. 108, 1595-1600.
27. Fechheimer, M. and Cebra, J. J. (1982) J. Cell Biol. 93, 261-268.